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Dated: 5/9/05 Signature: Paula Depelteau
(Maura Gallagher)
PAULA DEPELTEAU

Docket No.: SUPP-P01-016
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Brazelton et al

Application No.: 09/993,045 Group Art Unit: 1632

Filed: November 13, 2001 Examiner: Q. Janice Li

For: Methods for Treating Disorders of Neuronal Deficiency with Bone Marrow Derived Cells

MS RCE
Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 35 U.S.C. §1.132 OF TIMOTHY BRAZELTON

Sir:

Timothy Brazelton, Ph.D., hereby declares and states as follows:

1. I am a named inventor of the above-identified patent application, and of the subject matter described and claimed therein.

2. I am presently a Research Scientist at the Baxter Laboratory for Genetic Pharmacology, Stanford, CA. I conducted my doctoral research in the laboratory of Dr. Helen Blau at the

Stanford University Medical School. My curriculum vitae is attached to this Declaration as Exhibit BA.

3. Since the filing of the above-mentioned patent applications, additional data has been generated in Dr. Blau's laboratory that demonstrate the effectiveness of the techniques disclosed in the patent application. These data demonstrate that bone marrow derived cells, administered intravenously, ameliorate symptoms of Parkinson's disease in a well-established mouse model. The methods used and the data generated are described below.

4. We used a mouse model of Parkinson's disease. Parkinson's disease (PD) is a common neurodegenerative disorder characterized clinically by tremor, slowness of movement, stiffness, and postural instability. These clinical features are primarily attributable to the degeneration of nigrostriatal dopaminergic neurons in the substantia nigra (SN), loss of their projecting nerve fibers to the striatum, and depletion of the neurotransmitter dopamine. The neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) produces neurochemical and behavior deficits in when administered to mice, monkeys, and humans. MPTP-induced Parkinson's in mice is considered an excellent model of PD, one of few models for PD where the causative agent is known to induce similar Parkinsonian symptoms in humans and animals.

5. Bone marrow derived cells were prepared and administered as described below. Bone marrow was harvested from 8-10 week old, male transgenic mice that ubiquitously expressed an enhanced version of green fluorescent protein (GFP) driven with a B-actin promoter and a CMV enhancer. Briefly, donor mice were euthanized by cervical dislocation, immersed in 70% ethanol, and the skin was peeled back from a midline, circumferential incision. Large limb bones (femur, tibia, & humerus) were surgically isolated and placed in ice-cold of calcium and magnesium-free, Hank's balanced salt solution (HBSS, Irvine Scientific) with 2% FBS for up to 90 minutes. In a tissue culture hood, the tips of the bones were removed and a 25 gauge needle containing 1 mL of ice-cold HBSS with 2% FCS was inserted into the marrow cavity and used to wash the marrow out into a sterile culture dish. Marrow fragments were dissociated by titurating through the 25 gauge needle and the resulting suspension was filtered through sterile 70 μm nitex mesh. The filtrate was cooled on ice, spun for 5 minutes at 250 x g, and the pellet was resuspended in ice-cold HBSS with 2% FCS to $8x10^6$ nucleated cells per mL. Simultaneously,

the marrow of 8-10 week old, isogeneic (C57B/6, Jackson Laboratories, USA), recipient mice was ablated by lethal irradiation (two doses of 475 cGy, three hours apart). Within the 2 hours following lethal irradiation, each mouse received 125μL of the unfractionated BM suspension by tail vein injection.

6. MPTP (Sigma, USA) was administered in four intraperitoneal injections of 20mg/kg (free base) at 3 hour intervals for a total dose of 80 mg/kg. This dose was sufficient to induce biochemical and behavioral deficits along with dopaminergic cell loss.

7. Bone marrow transplantation (BMT) into recipient mice either before and after MPTP-induced neurodegeneration increased the number of tyrosine hydroxylase dopaminergic neurons in the substantia nigra and significantly increased dopamine transporter immunoreactivity in the striatum compared to non-transplanted, MPTP-treated mice. Importantly, there was significantly improved motor performance of the BM-transplanted, MPTP-injured mice as determined by rotorod and open field behavior tests. For the learned, accelerating rotorod test, mice are trained prior to the experiment to stay on a rotating rod that accelerates over time. For each rotorod test, mice are tested 3 times and the length of time on the rod is recorded.

8. Fig. 1, shown in Exhibit BB, illustrates the rotorod performance of three groups of mice. On day 0, mice received an injection of either MPTP or saline. The first rotorod test occurred on day 2. On day 6, one group of MPTP-treated mice were lethally irradiated and then received a whole bone marrow by tail vein injection. Mice were then tested on the rotorod on days 8, 15 and 20. The group of mice that received a BMT had a steady improvement in their rotorod performance in the 2 weeks following the BMT while the non-transplanted group's poor performance was maintained. Data is representative of 2 experiments. Saline group: n=10, BMT/MPTP: n=10, and MPTP: n=7. Data represents the mean time±SEM spent on the rotorod prior to falling. Student t tests for MPTP vs MPTP/BMT are p=0.03 and p=.0004 at days 15 and 20, respectively.

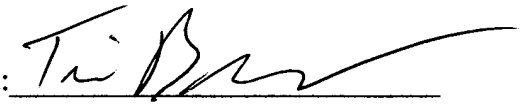
9. In an additional experiment, lethally irradiated mice received a transplant of whole bone marrow 8 weeks prior to MPTP-induced injury (Fig. 2, in Exhibit BB). Additional control groups included a group that received a BMT but no MPTP injection (BMT/saline) and a group that received head irradiation and MPTP injection to ensure that irradiation wasn't inhibiting the

response of cells in the CNS to the MPTP injection (Head irr/MPTP). While the BMT-transplanted group did demonstrate an acute loss of motor function in response to MPTP injection similar to that of non-transplanted mice (MPTP group), the BMT mice rapidly recovered and maintained their lost motor function. Figure 2 is representative of 3 experiments (n=7 for each group). The student t tests for the MPTP vs the BMT/MPTP groups are p=.02, p=.04, and $p<0.0001$ at days 8, 15, and 20, respectively.

10. BMT, therefore, improved both the pathological and functional measures of Parkinson's disease in this murine model and indicate that BMT can be used as a novel non-invasive, cell-based therapy for Parkinson's disease. These data demonstrate that the administration of bone marrow derived cells is effective to ameliorate symptoms of a neurodegenerative disease in a well-established animal model.

11. I further state that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 4-4-05

Signed: 

Dr. Timothy Brazelton